

AMENDMENTS

Amendments to the Specification

Please replace the paragraph at page 7, lines 1-6, with the following replacement paragraph:

Thus, as an example, nonapeptides potentially having the ability to bind to HLA-A1 would have one of the following sequences: Xaa-T-D-Xaa-Xaa-Xaa-L-Xaa-Y (SEQ ID NO: 86), Xaa-T-E-Xaa-Xaa-Xaa-L-Xaa-Y (SEQ ID NO: 87); Xaa-S-D-Xaa-Xaa-Xaa-L-Xaa-Y (SEQ ID NO: 88) or Xaa-S-E-Xaa-Xaa-Xaa-L-Xaa-Y (SEQ ID NO: 89) (Xaa indicating any amino acid residue). In a similar manner, sequences potentially having the ability to bind to any other HLA molecule can be designed.

Please replace the paragraph at page 19, lines 1-7, with the following replacement paragraph:

FIG. 7 illustrates HLA-35 binding of survivin-derived peptides and analysis of the peptide-mediated recovery of HLA-B35 molecules by survivin-derived peptides. Lysates of metabolically labeled T2-B35 cells were incubated at 4°C. in the presence of 50, 5, 0.5, 0.05 and 0.005 mM of peptide. The recovery of HLA-B35 was analyzed in an assembly assay and quantified subsequent to IEF-gel electrophoresis, using ImageGauge ~~phosphorimager~~ PHOSPHORIMAGER software (FUJI photo film Co., LTD., Japan). The C₅₀ value is the concentration of the peptide required for half-maximal binding to HLA-B35,

Please replace the paragraph at page 19, lines 25-29, with the following replacement paragraph:

FIG. 10 illustrates binding affinity of survivin-derived peptides to HLA-A1. Class I MHC heavy chain bands were quantified on a ~~phosphorimager~~ PHOSPHORIMAGER. The amount of stabilized HLA-A1 heavy chain is directly related to the binding affinity of the added peptide. The peptide-mediated recovery of HLA-A1 (arbitrary units) induced by 40, 4, 0.4, 0.04 μ M of Sur93-101 (line), Sur93T2 (square), Sur49-58 (circle) or Influenza A, PB1 591-599 (triangle),

Please replace the paragraph at page 23, lines 4-14, with the following replacement paragraph:

Peripheral vein blood samples from 4 patients diagnosed with CLL (designated CLL1-4) and blood samples from 6 normal individuals were collected into heparinised tubes. PBLs were isolated using ~~Lymphoprep~~ LYMPHOPREP separation and frozen in fetal calf serum (FCS) with 10% dimethylsulphoxide. Additionally, T lymphocytes from tumor-infiltrated lymph nodes were obtained from 6 melanoma patients (designated mel 1-6). Freshly resected lymph nodes were minced into small fragments, crushed to release cells into culture and cryopreserved. PBLs were available from 4 of the melanoma patients. All individuals included were HLA-A2 positive as determined by FACS analysis using the HLA-A2 specific antibody BB7.2. The antibody was purified from hybridoma supernatant. Patient samples were obtained from the State University Hospital, Herlev, Denmark. Informed consent was obtained from the patients prior to any of these measures.

Please replace the paragraph at page 24, lines 1-9, with the following replacement paragraph:

Assembly assays for binding of the synthetic peptides to class I MHC molecules metabolically labeled with [35S]-methionine were carried out as described (12,13). The assembly assay is based on stabilization of the class I molecules after loading of peptide to the peptide transporter deficient cell line T2. Subsequently, correctly folded stable MHC heavy chains are immunoprecipitated using conformation-dependent antibodies. After IEF electrophoresis, gels were exposed to ~~phosphorimager~~ PHOSPHORIMAGER screens, and peptide binding was quantified using the Imagequant ~~phosphorimager~~ PHOSPHORIMAGER program (Molecular Dynamics, Sunnyvale, Calif.).

Please replace the paragraph at page 24, lines 25-39, with the following replacement paragraph:

The ELISPOT assay used to quantify peptide epitope-specific interferon- γ releasing effector cells was performed as in (16). Briefly, nitrocellulose bottomed 96-well plates (MultiScreen MAIP N45, Millipore, Hedeusene, Denmark) were coated with anti-IFN- γ antibody (1-D1K, Mabtech, Nacka, Sweden). The wells were washed, blocked by AIM V medium, and cells were added in duplicates at different cell concentrations. Peptides were then added to each well and the plates were incubated overnight. On the following day, medium was discarded and the wells were washed prior to addition of biotinylated secondary antibody (7-B6-1-Biotin, Mabtech). The plates were incubated for 2 hours, washed and Avidin-enzyme conjugate (AP-Avidin, Calbiochem, Life Technologies) was added to each well. Plates were incubated at RT for 1 hour and the enzyme substrate NBT/BCIP (Gibco, Life Technologies) was added to each well and incubated at room temperature for 5-10 min. The reaction was terminated by washing with tap water upon the emergence of dark purple spots. The spots were counted using the ~~AlphaImager~~ ALPHAIMAGER System (Alpha Innotech, San Leandro, Calif. USA) and the peptide specific CTL frequency could be calculated from the numbers of spot-forming cells. The assays were all performed in duplicate for each peptide antigen.

Please replace the paragraph at page 27, lines 30-41, with the following replacement paragraph:

The ELISPOT assay was used to quantify peptide epitope-specific IFN- γ releasing effector cells and has been described previously (39). Briefly, nitrocellulose bottomed 96-well plates (MultiScreen MAIP N45, Millipore) were coated with an anti-IFN- γ antibody (1-D1K, Mabtech, Sweden) and non-specific binding was blocked using AIM V (GibcoBRL, Life Technologies Inc., Gaithersburg, Md., USA). Lymphocytes were added at different cell concentrations together with the specific peptides and T2 cells and incubated overnight at 37°C. Following two washes the biotinylated detection antibody (7-B6-1-Biotin, Mabtech) was added. Specific binding was visualised using alkaline phosphatase-avidin together with the respective substrate (GibcoBRL). The reaction was terminated upon the appearance of dark purple spots, which were quantitated using the ~~AlphaImager~~ ALPHAImAGER System (Alpha Innotech, San Leandro, Calif., USA). The peptides used for the ELISPOT were Sur1, Sur9 and the Sur1 analogue peptide Sur1M2 as described in Example 1.

Please replace the paragraph at page 30, lines 30-38, with the following replacement paragraph:

Peripheral vein blood samples from cancer patients were collected, PBLs were isolated using ~~Lymphoprep~~ LYMPHOPREP separation, HLA-typed (Department of Clinical Immunology, University Hospital, Copenhagen) and frozen in FCS with 10% DMSO. Ten HLA-B35 positive patients were selected for further analysis. These patients suffered from melanoma, CLL, follicular lymphoma (FL), diffuse large B-cell lymphomas (DLBCL) and Multiple Myeloma (MM), respectively. At the time blood samples were collected patients had not been

medically treated within the previous four months. Additionally, tumor-infiltrating lymphocytes (TIL) isolated from lymph nodes were collected from three of the melanoma patients and frozen in FCS with 10% DMSO.

Please replace the paragraph at page 31, lines 9-18, with the following replacement paragraph:

The assembly assay described in Examples 1 and 2 was used to measure binding affinity of the synthetic peptides to HLA-B35 molecules metabolically labeled with [S35]methionine. Briefly, the assay is based on peptide-mediated stabilization of empty HLA molecules released, upon cell lysis, from the TAP deficient cell line T2, stably transfected with HLA-B35 (kindly provided by Dr J. Haurum, Symphogen ApS, Lyngby, Denmark). Stably folded HLA-molecules were immunoprecipitated using the conformation-dependent mAb W6/32. The HLA molecules were separated by IEF electrophoresis, gels were exposed to ~~phosphorimager~~ PHOSPHORIMAGER screens (Imaging plate, FUJI photo film Co., LTD., Japan), analyzed and the amount of correctly folded HLA molecules were quantified using ImageGauge ~~phosphorimager~~ PHOSPHORIMAGER software (FUJI photo film Co., LTD., Japan).

Please replace the paragraph at page 35, line 41 to page 36, line 7, with the following replacement paragraph:

Patient samples were received from the University of Wurzburg, Germany and the University Hospital in Herlev, Denmark. Informed consent was obtained from the patients prior to any of these measures. Tissue typing was conducted at Department of Clinical Immunology, University Hospital, Copenhagen, Denmark. Peripheral blood lymphocytes (PBL) from cancer

patients with melanoma, mamma carcinoma, and chronic lymphocytic leukemia (CLL) were isolated using ~~Lymphoprep~~ LYMPHOPREP separation and frozen in fetal calf serum (FCS) with 10% dimethylsulphoxide. Furthermore, T lymphocytes from primary lesions and from tumor infiltrated lymph nodes from melanoma patients were obtained. Freshly resected tumor tissue was minced into small fragments, and crushed to release tumor-infiltrating lymphocytes (TIL) for cryopreservation.

Please replace the paragraph located on page 37, line 36 extending to page 38, line 20, with the following replacement paragraph:

The amino acid sequence of the survivin protein was screened for the most probable HLA-A1 nonamer or deca-mer peptide epitopes, using the main HLA-A1 anchor residues, aspartic acid (D), glutamic acid (E) at position 3 and tyrosine (Y), phenylalanine (F) at the C-terminus. Accordingly, six survivin-derived peptides were synthesized and examined for binding to HLA-A1 (table 4). Additionally, the two peptides Sur38-46 (MAEAGFIHC)(SEQ ID NO:23) and Sur47-56 (PTENEPDLAQ) (SEQ ID NO:25) was included, in spite they only contain one of the main anchors, since both were identified as possible good binders by the predictive algorithm by Rammensee et al. ~~available at <http://syfepeithi.bmi-heidelberg.com/>~~. C₅₀ values were estimated for each peptide as the peptide concentration needed for half maximal stabilization of HLA-A1 (table 4). However, only one of these peptides Sur92-101 (QFEELTLGEF) (SEQ ID NO:27) bound with almost similar high affinity as a known positive control epitope from the Influenza A protein, basic polymerase 1 (PB1) (VSDGGPNLY) as exemplified in FIG. 10. Sur93-101 (FEELTLGEF) (SEQ ID NO:24) had a low binding affinity for HLA-A1, whereas none of the other peptides analyzed bound to HLA-A1 (Table 4). Consequently, we synthesized a number of analogue peptides in which better anchor residues replaced the natural amino acids. We modified the two peptides Sur38-46 (MAEAGFIHC) (SEQ ID NO:23) and Sur47-56 (PTENEPDLAQ) (SEQ ID NO:25) introducing tyrosine (Y) instead of cysteine (C) or glutamine (Q) respectively at the C-terminus. Both of the modified peptides bound strongly to HLA-A1

(table 4). Additionally, we substituted the amino acids at position 2 with the auxiliary anchors threonine (T) or serine (S) in the two peptides Sur92-101 and Sur93-101. These modifications did not have a positive effect of the binding of Sur92-101 to HLA-A1. In contrast, the Sur93T2 (FTELTGGEF) (SEQ ID NO:36) bound with high affinity to HLA-A1 (Table 4). FIG. 10 illustrates the binding of the native low affinity peptide Sur93-101, the high affinity modified peptide Sur93T2 and the non-binding peptide Sur49-58 as compared to the positive control epitope from influenza. Finally, we modified Sur14-22, Sur34-43, Sur49-58, Sur51-59, Sur92-101, and Sur93-101 with tyrosine (Y) at the C-terminus, however this did not improve binding affinity to HLA-A1 for any of these peptides (data not shown).

Please replace the paragraph at page 44, lines 17-36, with the following replacement paragraph:

All clinical procedures were in accordance with the Declaration of Helsinki and all patients provided informed consent prior to therapy. Stage IV cutaneous or uveal melanoma patients were eligible when their disease was progressive despite at least two different chemo-, immuno, or chemoimmunotherapies. In addition, a patients had to be 18 years or older, express HLA*0201, and suffer from measurable disease validated by cranial, thoracic and abdominal computed tomography scans. Patients' Karnofsky index had to be 60% or better. No systemic chemo-, and/or immunotherapy was allowed within 4 week prior to vaccination. Important exclusion criteria were evidence of CNS metastases, active autoimmune or infectious diseases, pregnancy and lactation, as well as significant psychiatric abnormality. Peptide pulsed dendritic cells were generated as previously described (82). Briefly, PBMCs from leukapheresis were isolated on ~~Lymphoprep~~TM LYMPHOPREP (Nycomed Pharma), frozen in aliquots and stored in liquid nitrogen. One week prior to vaccination, PBMCs were thawed, washed and cultured in medium containing gentamycin, glutamine and heat inactivated autologous plasma. On day 1 and 5, IL-4 and GM-CSF were added. To differentiate mature DCs, TNF- γ and prostaglandin E2 were added on day 6. On day 7, cells displaying phenotypical and morphological

characteristics of mature DCs, i.e. a veiled appearance and =75% CD83 expression, were pulsed with a modified survivin-derived HLA-A2 restricted survivin₉₆₋₁₀₄ epitope, LMLGEFLKL (SEQ ID NO 10)(Clinalfa, Switzerland)¹⁴. Cells were only used for vaccination if microbial tests of samples taken from cultures on days 1 and 5 proved to be sterile.

Please replace the paragraph at page 45, lines 5-22, with the following replacement paragraph:

CT scans were performed prior to vaccination and every three months thereafter or in case of severe clinical signs of disease progression. Immunological responses were monitored by the ELISPOT assay, using PBMCs obtained every three months, to detect survivin₉₆₋₁₀₄ specific IFN- γ release. To extend the sensitivity of the ELISPOT assay, PBMCs were stimulated once in vitro at a concentration of 1×10^6 cells per ml in 24-well plates (Nunc, Denmark) in X-vivo medium (Bio Whittaker, Walkersville, Md.), supplemented with 5% heat-inactivated human serum and 2 mM of L-glutamine in the presence of 10 μ M of peptide. Two days later, 40 IU/ml recombinant interleukin-2 (IL-2) (Chiron, Ratingen, Germany) were added. After 10 days the cells were tested for reactivity. To this end, nitrocellulose bottomed 96-well plates (MultiScreen MAIP N45, Millipore, Glostrup, Denmark) were coated with an anti-IFN- γ antibody (1-D1K, Mabtech, Sweden). Lymphocytes were added at 10^4 - 10^5 cells in 200 μ l X-vivo medium per well together with 10^4 T2-cells and the relevant peptides at a final concentration of 2 μ M. After an overnight incubation at 37°C and two washes, the biotinylated detection antibody (7-B6-1-Biotin, Mabtech, Sweden) was added; its specific binding was visualised using alkaline phosphatase-avidin together with the respective substrate (GibcoBRL). The reaction was terminated upon the appearance of dark purple spots, which were quantitated using the ~~AlphaImager~~ ALPHAIMAGER System (Alpha Innotech, San Leandro, Calif., USA).